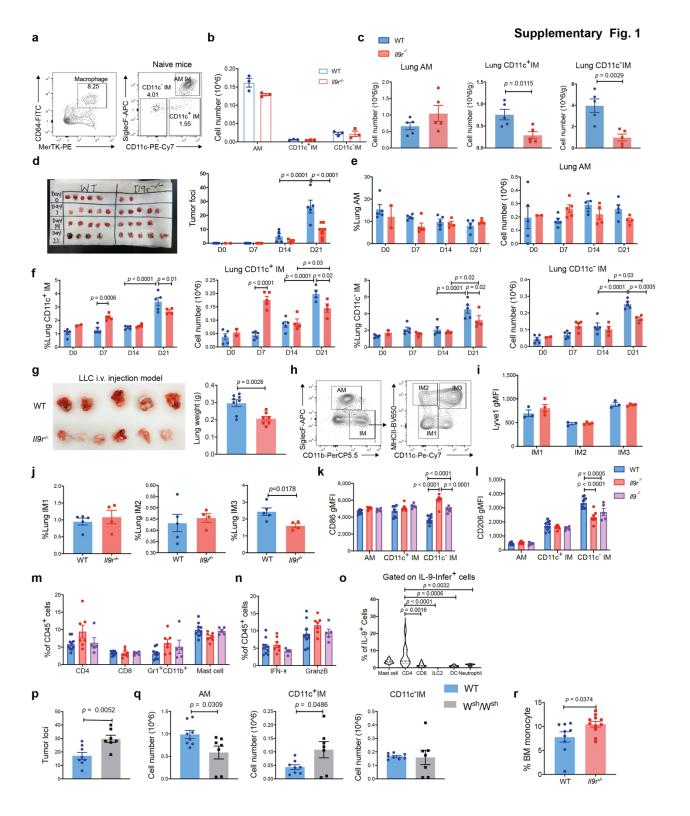
Murine pulmonary interstitial macrophages mediate the pro-tumorigenic effects of IL-9

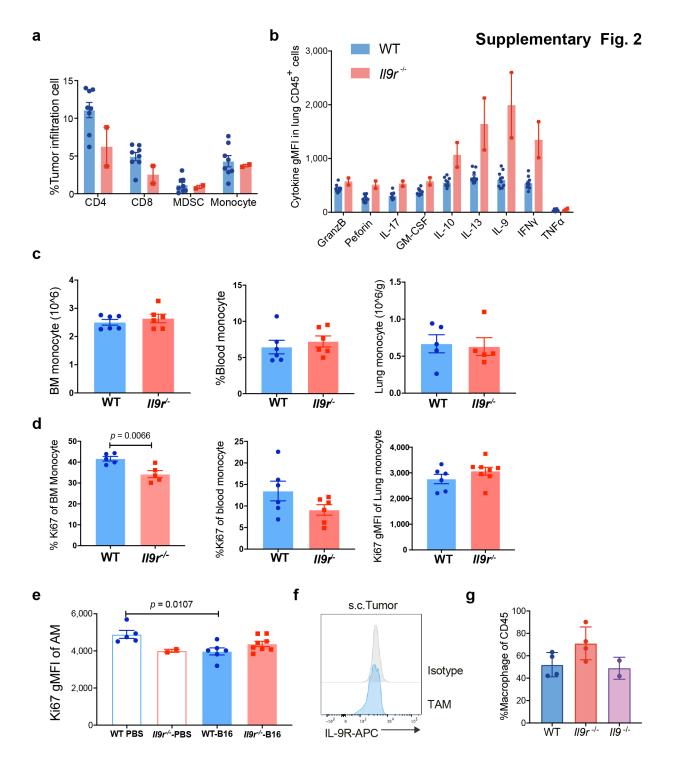
Yongyao Fu¹, Abigail Pajulas¹, Jocelyn Wang¹, Baohua Zhou², Anthony Cannon¹, Cherry Cheuk Lam Cheung¹, Jilu Zhang¹, Huaxin Zhou³, Amanda Jo Fisher³, David T Omstead⁴, Sabrina Khan⁴, Lei Han², Jean-Christophe Renauld⁵, Sophie Paczesny⁶, Hongyu Gao⁷, Yunlong Liu⁷, Lei Yang², Robert M Tighe⁸, Paula Licona-Limón⁹, Richard A. Flavell¹⁰, Shogo Takatsuka¹¹, Daisuke Kitamura¹¹, Jie Sun¹², Basar Bilgicer⁴, Catherine R Sears³, Kai Yang², Mark H Kaplan¹, *

Supplementary Figures and Tables



Supplementary Fig. 1. IL-9 promotes tumor growth by altering lung macrophage populations.

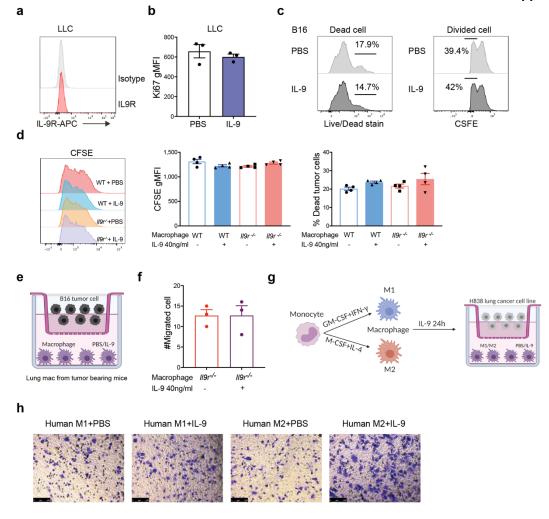
- (a-b), Flow cytometric analysis of lung macrophage numbers from naïve mice (n = 3 mice).
- (c), Flow cytometric analysis of lung macrophage numbers from tumor bearing mice (n = 5 mice).
- (d-f), Tumor bearing mice were analyzed after one, two or three weeks of tumor injection. Tumor growth (d) and lung macrophage numbers (e and f) were analyzed (n = 5 mice for WT group, n = 2 mice for d0 $Il9r^{-/-}$ group, n = 5 for d7 $Il9r^{-/-}$ group, n = 4 mice for d14 and d21 $Il9r^{-/-}$ group).
- (g), Tumor growth and lung weight in LLC metastasis model (n = 8 mice for WT group, n = 7 mice $Il9r^{-/-}$ group).
- (h), Dot plot analysis of IM subsets from B16 tumor bearing mice.
- (i), Lyve1 expression in IM subsets from B16 tumor bearing mice (n = 3 mice for WT group, n = 4 mice $ll9r^{-/-}$ group).
- (j), IM subset analysis from B16 tumor bearing mice (n = 5 mice for WT group, n = 4 mice $Il9r^{-/-}$ group).
- (k-n), B16 melanoma cells were intravenously injected into the mice. CD86 expression (k) (n = 9 mice for WT group, n = 7 mice $Il9r^{-/-}$ group, n = 5 mice $Il9r^{-/-}$ group) and CD206 expression (l) (n = 10 mice for WT group, n = 7 mice $Il9r^{-/-}$ group, n = 5 mice $Il9r^{-/-}$ group), Immune cells and cytokine production from CD45⁺ cells (m-n) were analyzed by flow cytometry (n = 10 mice for WT group, n = 7 mice $Il9r^{-/-}$ group, n = 5 mice $Il9r^{-/-}$ group).
- (o), IL-9 reporter (Infer) mice were injected with B16 tumor, IL-9 producing cells were analyzed on day 21 (n = 8 mice for mast cell, DC and neutrophil, n = 9 mice for CD4 cell, n = 10 for CD8 and ILC2).
- (p-q), Tumor growth (p) and lung macrophage numbers (q) were analyzed on day 21 (n = 8 mice for WT group, n = 7 mice for W^{sh}/W^{sh} group).
- (r), Percentages of bone marrow monocytes from LLC intravenously injected tumor bearing mice were analyzed by flow cytometry (n = 9 mice for WT group, n = 11 mice $Il9r^{-/-}$ group).
- Data are the mean \pm SEM. Unpaired two-tailed Student t-test was used for comparison in c, j, p, q and r. Two-way ANOVA with Sidak's multiple comparisons was used for comparison in e, f, k and l. One-way ANOVA with a Dunnett's multiple comparison test was used for multiple comparisons in o.



Supplementary Fig. 2. IMs are the TAMs that respond to IL-9 and promote tumor growth (a-b), LLC cells were injected to the lung, Tumor-infiltrating immune cells were analyzed by flow cytometry (a) (n = 9 mice for WT group, n = 2 mice for $Il9r^{-/-}$ group). (b), Cytokine production was analyzed (n = 9 mice for WT GranzB, Perforin, IL-17, and GM-CSF, n = 10 mice for WT IL-10, IL-13, IL-9, IFNy and TNF α group, n = 2 mice $Il9r^{-/-}$ group).

- (c), Monocytes from bone marrow, blood and lung were analyzed 21days after B16 tumor injection (n = 6 mice for groups in left and middle panel, n = 5 mice for groups in right panel).
- (d), Ki67 expression in monocytes from bone marrow, blood and lung were analyzed 21 days after B16 tumor injection (n = 5 mice for groups in left panel, n = 6 mice for groups in middle panel, n = 6 for WT group and n = 8 mice for $ll9r^{-/-}$ group in right panel).
- (e), Ki67 expression of lung AMs were analyzed 21 days after B16 tumor injection (n = 4 mice for WT PBS group, n = 2 mice for for $ll9r^{-/-}$ PBS group, n = 6 for WT-B-16 group and n = 8 mice for $ll9r^{-/-}$ B16 group).
- (f-g), IL-9R expression in TAMs (f) and macrophage percentage (g) from s.c. B16 tumor bearing mice (n = 4 mice for WT and $ll9r^{-/-}$ group, n = 2 mice for $ll9^{-/-}$ group).

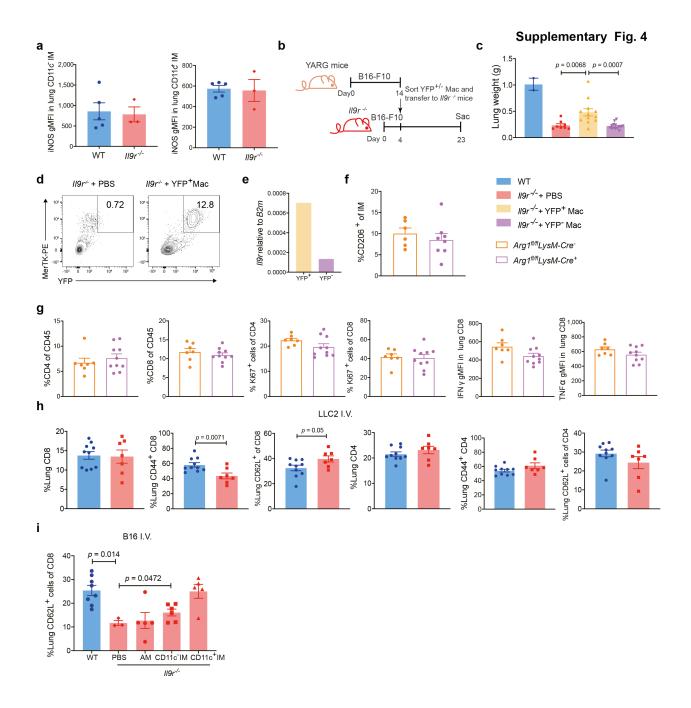
Data are the mean \pm SEM. Unpaired two-tailed Student t-test was used for comparison in d-e.



Supplementary Fig. 3. IL-9 promotes macrophage mediated tumor migration

- (a), IL-9R expression on LLC cancer cell line was analyzed.
- (b), LLC cancer cells were treated with IL-9 for 24hs. Ki67 expression was analyzed (n = 3 independent wells).
- (c), B16 cells were treated with IL-9 for 48hs. Cell death and proliferation was analyzed.
- (d), Total lung macrophages were isolated from LLC tumor bearing mice and cocultured with LLC cells for 72 hours. LLC cell proliferation and cell death were analyzed by flow cytometry (n = 4 mice).
- (e-f), Total lung macrophages were isolated from tumor bearing mice and plated in the lower chamber of the transwell with or without IL-9, tumor cells were plated in the upper chamber (e). (f), Migrated cells were visualized with crystal violet staining (n=3 mice).
- (g-h), Human monocytes were cultured with GM-CSF or M-CSF for polarizing into M1 or M2 macrophage for 5 days before IFN- γ or IL-4 were respectively added to the cultures for 48hs. IL-9 was added for an additional 24 hours. The migration assay was performed (g) and human 838 cells were stained with crystal violet (h), Scale bar = 100 μ m.

Data are the mean \pm SEM. Unpaired two-tailed Student t-test was used for comparison in b and f. One-way ANOVA with a Dunnett's multiple comparison test was used for multiple comparisons in d.

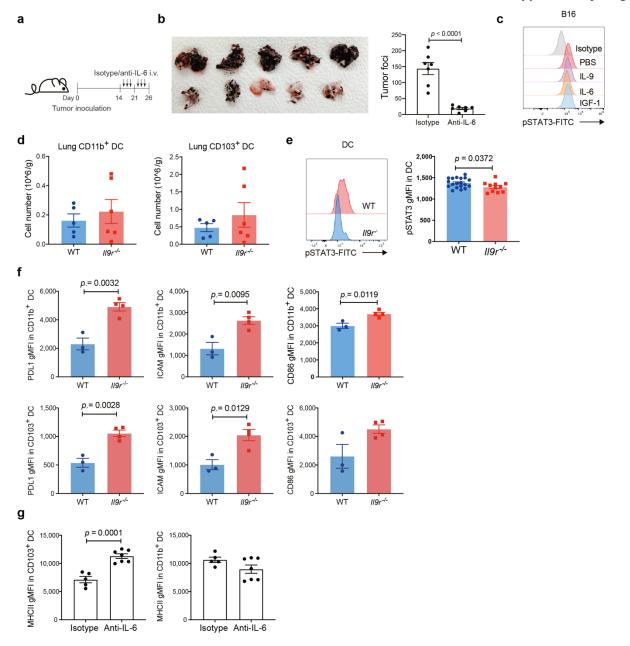


Supplementary Fig. 4. IL-9 impacts lung macrophage function by regulating Arg1 expression.

- (a) iNOS expression from IMs were analyzed by flow (n= 5 mice for WT group, n = 3 for $Il9r^{-/-}$ group).
- (b-e), YARG mice were injected with B16 cells, and lung Arg1^{+/YFP} macrophages were sorted on day 14. Cells were intravenously injected into $Il9r^{-/-}$ mice 4 days after tumor injection (b). Lung weights were measured on day 23 (c) (n = 2 mice for WT group, n = 8 mice for $Il9r^{-/-}$ + PBS group,

- n = 12 mice for $Il9r^{-/-}$ + YFP⁺ Mac group, n = 14 mice for $Il9r^{-/-}$ +YFP⁻ Mac group). Arg1 expression was analyzed in macrophages by flow cytometry (d). Il9r expression was analyzed from FACS sorted donor macrophages (e).
- (f), CD206 expression in IMs from $Arg \, l^{fl/fl} \, LysM$ - Cre^+ mice or littermate control mice (n = 6 mice for $Arg \, l^{fl/fl} \, LysM$ - Cre^- group, n = 8 mice for $Arg \, l^{fl/fl} \, LysM$ - Cre^+ group).
- (g), Percentages of lung CD4 and CD8 T cells, Ki67 expression in T cells and cytokine expression from CD8 cells were analyzed by flow cytometry (n = 7 mice for $Arg I^{fl/fl} LysM-Cre^-$ group and n = 10 mice for $Arg I^{fl/fl} LysM-Cre^+$ group).
- (h), Percentages of lung CD4 and CD8 T cells, CD44⁺ and CD62L⁺ T cells were analyzed by flow (n= 10 mice for WT group, n = 7 mice for $ll9r^{-/-}$ group).
- (i), Macrophage adoptive transfer experiment was performed as shown in Fig. 5d. Lung CD62L+ CD8 T cells were analyzed (n =8 mice for WT group, n = 3 mice for PBS group, n = 5 mice for AM and CD11c⁺ IM groups, n = 6 mice for CD11c⁻ IM group).

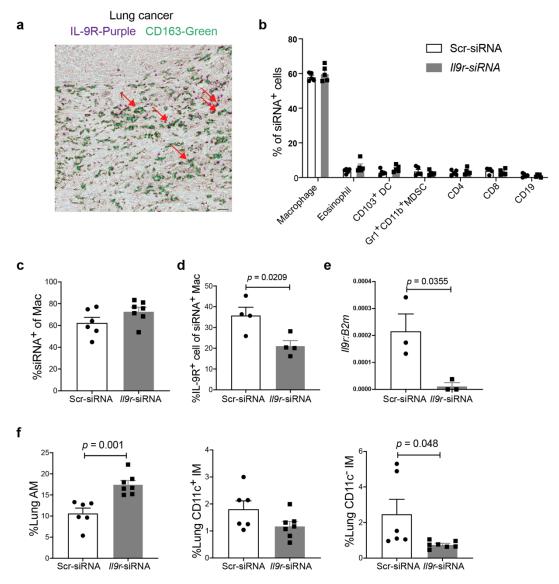
Data are the mean \pm SEM. One-way ANOVA with a Dunnett's multiple comparison test was used for multiple comparisons in c and i. Unpaired two-tailed Student t-test was used for comparison in h.



Supplementary Fig. 5. IL-9 induces IL-6 expression in Arg1 expressing IMs. (a-b), WT tumor bearing mice were treated with isotype antibody or anti-IL-6 (a) and tumor growth was analyzed (b) (n = 7 mice).

(c), B16 cells were stimulated for 60 minutes and pSTAT3 expression was analyzed. (d-f), DC numbers (d) (n = 5 mice for WT group, n = 6 for $Il9r^{-/-}$ group), pSTAT3 expression (e) (n = 19 mice for WT group, n = 11 mice for $Il9r^{-/-}$ group), and activation markers (f) (n = 3 mice for WT group, n = 4 mice for $Il9r^{-/-}$ group) were analyzed from B16 tumor bearing mice. (g), Mice were treated as shown in b, MHCII expression from lung DCs was analyzed by flow cytometry (n = 5 mice for isotype group, n = 7 mice for anti-IL-6 group).

Data are the mean \pm SEM. Unpaired two-tailed Student t-test was used for comparison.

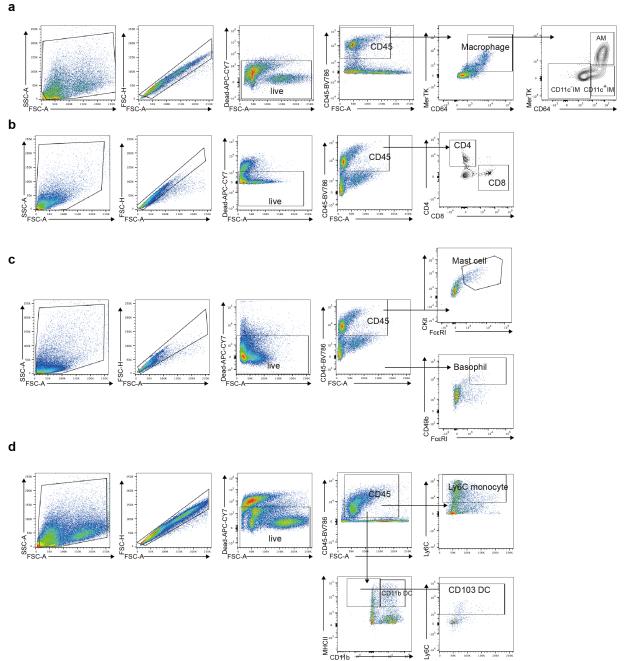


Supplementary Fig. 6. Therapeutic targeting of the IL-9-macrophage axis prevents lung cancer growth.

- (a), Immunohistochemistry staining of CD163 and IL-9R in lung cancer patient tissue, Scale bar = $100 \ \mu m$.
- (b-f), WT mice were intravenously injected with B16 tumor cell line. Seven days after tumor inoculation, tumor-bearing mice were intravenously injected with nanoparticle-siRNA complexes every 72 hours. Scr/*Il9r*-siRNA was conjugated with Alexa Flour 555. Nanoparticles were tagged with SIRPα peptide. (b-c) Lung cells that had nanoparticle-siRNA complex uptake were analyzed by flow cytometry (n = 5 mice for groups in b, n = 6 mice for Scr-siRNA group and n = 7 for *Il9r*-siRNA group in c). (d), IL-9R expression in siRNA⁺ (Alexa Flour 555) lung macrophages were analyzed by flow (n = 4 mice). (e), siRNA⁺ (Alexa Flour 555) lung macrophages were sorted by gating on Alexa Flour 555⁺ MerTK⁺ CD64⁺ live cells. Gene expression was analyzed (n = 3 mice). (f) Lung macrophages were analyzed by flow cytometry. (n = 6 mice for Scr-siRNA group and n

= 7 for Il9r-siRNA group in the middle and right panels). Data are the mean \pm SEM. Unpaired two-tailed Student t-test was used for comparison.

Supplementary Fig. 7



Supplementary Fig. 7. Gating strategies used for identifying different cell types

Gating strategies for identifying lung macrophages (a), T cells (b), mast cell and basophil (c), monocytes and DC (d). Strategy for macrophages was used in Figures 2c-f, h, i, k, Figure 4d, g, h, k, Figure 5b, c, Figure 7b-e, i, l, Figure 8e-j, Figure 9i, k, l and Supplementary Figures 1a-c, e-f, h, i-l, q-r, 2e-g, 4a, d, f, and 6c-f. T cell gating was used in Supplementary 4g-i. Monocyte gating was used in Supplementary 2c-d. DC gating was used in Supplementary 5c-g. All strategies were used for Figures 2c and f, 7l, and Supplementary figures 1m-o, 2a-b, and 6b.

Supplementary Tables

Supplementary Table 1. Lung cancer patient sample information related to Fig. 9c.

Description/diagnosis information	Gender	Age	Catalog Number
Lung, within normal limits	Female	74	CR561260
Lung, within normal limits	Female	76	CR563059
Lung, within normal limits	Male	72	CR559058
Lung, within normal limits	Female	76	CR562690
Lung, within normal limits	Male	46	CR562269
Lung, within normal limits	Female	55	CR560513
Lung, within normal limits	Male	45	CR562257
Lung, within normal limits	Male	72	CR559080
Lung, right upper lobe, adenocarcinoma of lung	Female	72	CR561124
Lung, carcinoma of lung, non-small cell	Male	65	CR561590
Lung, right upper lobe, adenocarcinoma of lung	Female	46	CR560308
Lung, carcinoma of lung, squamous cell, metastatic	Female	63	CR561521
Lung, carcinoma of lung, squamous cell	Male	57	CR562068
Lung, carcinoma of lung, squamous cell	Female	70	CR562209
Lung, right lower lobe, adenocarcinoma of lung	Male	72	CR562443
Lung, carcinoma of lung, squamous cell	Female	77	CR562025

Supplementary Table 2. Lung cancer patient sample information related to Fig. 9d.

Description/diagnosis information	Gender	Age
Healthy control	Female	53
Healthy control	Female	31
Healthy control	Female	24
Healthy control	Female	22
Healthy control	Female	47
Healthy control	Male	66
Healthy control	Female	65
Lung cancer	Male	70
Lung cancer	Female	64
Lung cancer	Female	31
Lung cancer	Female	47
Lung cancer	Female	53

Supplementary Table 3. Lung cancer patient sample information related to Fig. 9f-g.

Description/diagnosis information	Gender	Age	Catalog Number
Lung, within normal limits	Female	71	CS700238
Lung, within normal limits	Female	61	CS702878
Lung, within normal limits	Female	66	CS700243
Lung, within normal limits	Male	68	CS711408
Lung, within normal limits	Female	49	CS706500
Lung, within normal limits	Male	54	CS809755
Lung, within normal limits	Male	61	CS804028
Lung carcinoma of lung, squamous cell	Male	74	CS708748
Lung; Adenocarcinoma of lung	Male	52	CS707844
Lung; Carcinoma of lung, squamous cell	Male	81	CS704553
Lung; Carcinoma of lung, pleomorphic	Female	71	CS716372
Lung: right upper lobe; Carcinoma of lung, non-small cell, metastatic (EGFR+)	Male	51	CS708072
Lung: right middle lobe; Carcinoma of	Female	70	CS813393
lung, small cell, metastatic			
Lung; Carcinoma of lung, squamous cell,	Male	74	CS702498
metastatic			

Supplementary Table 4. Key resource table.

REAGENT or RESOURCE	SOURCE	IDENTIFIER	Dilution
Antibodies			
Anti-Mouse CD11b-PerCP-Cy5.5 (clone M1/70)	eBioscience	Cat#:45-0112-82	1:200
Anti-Mouse CD11c-PE-Cy7 (clone N418)	eBioscience	Cat#:25-0114-82	1:400
Anti-Mouse CD11c-BV650 (clone N418)	BioLegend	Cat#:117339	1:400
Anti-Mouse CD4-PE-Cy7 (clone GK1.5)	BioLegend	Cat#:100422	1:200
Anti-Mouse c-Kit-APC (clone 2B8)	BioLegend	Cat#:105811	1:200
Anti-Mouse CD49b-PE (clone HMα2)	BioLegend	Cat#:103506	1:200
Anti-Mouse Ki67- PE-eFluor 610 (clone SolA15)	Invitrogen	Cat#:61-5698-82	1:400
Anti-Mouse CCR7-Alexa Fluor 700 (clone 4B12)	eBioscience	Cat#:56-1971-82	1:200
Anti-Mouse Arg1-APC (clone A1exF5)	Invitrogen	Cat#:17-3697-82	1:200
Anti-Mouse IL-6-PE (clone MP5-20F3)	BD Biosciences	Cat#:554401	1:200
Anti-Mouse CD206-APC (clone C068C2)	BioLegend	Cat#:141708	1:200
Anti-Mouse CD45.1-BV786 (clone A20)	BD Biosciences	Cat#:740889	1:200
Anti-Mouse CD45.1-PerCP5.5 (clone A20)	BioLegend	Cat#:110728	1:200
Anti-Mouse CD45.2-PE-Cy7 (clone 104)	Invitrogen	Cat#:25-0454-82	1:200
Anti-Mouse CD115-APC (clone AFS98)	BioLegend	Cat#:135510	1:200
Anti-Mouse CCR2-BV786 (clone 475301)	BD Biosciences	Cat#:747966	1:200
Anti-Mouse I-A/I-E-BV421 (clone M5/114.15.2)	BioLegend	Cat#:107631	1:400
Anti-Mouse CD103-BV421 (clone M290)	BD Biosciences	Cat#:557495	1:200
Anti-Mouse Ly-6C-BV510 (clone HK1.4)	BioLegend	Cat#:128033	1:400
Anti-Mouse FceR1-PE-Cy7 (clone MAR-1)	BioLegend	Cat#:134317	1:200
Anti-Mouse Foxp3-FITC (clone MF23)	BD Biosciences	Cat#:560403	1:200
Anti-Mouse IFN-γ-PerCP-Cy5.5 (clone XMG 1.2)	eBioscience	Cat#:45-7311-82	1:200
Anti-Mouse IL-17A-PE-Cy7 (clone eBio17B72)	eBioscience	Cat#:25-7177-82	1:200
Anti-Mouse Ly6G-FITC (clone RB6-8C5)	BD Biosciences	Cat#:553126	1:200
Anti-Mouse SiglecF-BV605 (clone E50-2440)	BD Biosciences	Cat#: 740388	1:200
Anti-Mouse SiglecF-AF647 (clone E50-2440)	BD Biosciences	Cat#: 562680	1:200
Anti-Mouse CD64-FITC (clone X54-5/7.1)	BioLegend	Cat#: 139316	1:200
Anti-Mouse CD64-BV421 (clone X54-5/7.1)	BioLegend	Cat#: 139309	1:200
Anti-Mouse CD64-BV711 (clone X54-5/7.1)	BioLegend	Cat#: 139311	1:200
Anti-Mouse MerTK-PE (clone 2B10C42)	BioLegend	Cat#: 151506	1:200
Anti-Mouse MerTK-APC (clone 2B10C42)	BioLegend	Cat#: 151508	1:200
Anti-Mouse Gr1-APC (clone RB6-8C5)	BioLegend	Cat#: 108412	1:800
Anti-Human CD14-AF-700 (clone HCD14)	BioLegend	Cat#: 325605	1:200
Anti-Human IL-9R-PE (clone AH9R7)	BioLegend	Cat#: 310403	1:100
Biotin anti-mouse CD170 (SiglecF) Antibody (S17007L)	BioLegend	Cat#: 155512	1:200
Anti-Mouse Lyve1-APC	Thermo Fisher	Cat#: PA5-22782	1:200
Anti-Mouse Ly6G-PE-TexRed	Thermo Fisher	Cat#: 61-9668-82	1:200
Anti-Mouse MHCII-BV650	BC Bioscience	Cat#: 563415	1:400
Anti-Mouse IL-9R-APC	BioLegend	Cat#: 158806	1:100

Anti-Mouse CD86-FITC (clone GL-1) BioLegend Cat# 105006 1:200 Anti-Mouse CD86-FITC (clone GL-1) BioLegend Cat# 105006 1:200 Anti-Mouse CD86-FITC (clone GL-1) BioLegend Cat# 140346 1:200 Anti-Mouse CAt IgC11, Isotype Ctrl FITC Rat Anti-Mouse IgC31 BioLegend Cat#: 400412 1:200 FITC Rat Anti-Mouse IgC31 BioLegend Cat#: 553443 1:200 FITC anti-STAT3 Phospho (Tyr705) BioLegend Cat#: 553443 1:200 FITC anti-STAT3 Phospho (Tyr705) BioLegend Cat#: 651020 1:200 Anti-Human IL-9R Polychonal Antibody Thermo Fisher Cat#: 105220 1:50 Anti-Human CD68 antibody [PG-M1] BioLegend Cat#: 105220 1:50 Anti-Human IL-9R Polychonal Milteryl Biotec Cat#: 105220 1:50 Chemicals, Peptides, and Recombinant Proteins Collagenase D Milteryl Biotec Cat#: 130-100-629 CD14 MicroBeads UltraPure, human Milteryl Biotec Cat#: 130-100-629 CD14 MicroBeads UltraPure, human Milteryl Biotec Cat#: 130-100-629 Recombinant Murine IL-4 20ug Peprotech Cat#: 200-04 Recombinant Human II4 Protein R&D systems Cat#: 200-04 Recombinant Human II9 Protein R&D systems Cat#: 200-04 Recombinant Human II4 Peprotech Cat#: 200-04 Recombinant Human II4 Peprotech Cat#: 200-04 Recombinant Human III6 Protein R&D systems Cat#: 186006 1 Recombinant Human III6 Peprotech Cat#: 200-04 Recombinant For Systems Cat#: 08-833-6 Recombinant For Systems				
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Experimental Models: Cell Lines		Biolgend	Cat#: 431304	
	Experimental Models: Cell Lines			

B16-F10	Laboratory of I Yang	Or. 1	Dario Vignali and Dr. Kai
LLC (LL/2-Luc2)	ATCC		CRL-1642-LUC2
H838	ATCC		CRL-5844
Oligonucleotides			
Primers for mRNA expression, see Supplementary Table 6			
<i>Il9r</i> -siRNA for Figure 8	ThermoFisher		Cat#S201546
Scr-siRNA for Figure 8	ThermoFisher		Cat#4457289
Software and Algorithms			
GraphPad Prism V_8.0 and V_7.0	GraphPad Software		http://www.graphpad.c om
FlowJo (version 10.7.1)	LLC		http://www.flowjo.com
ImageJ V1.53a	NIH software		https://imagej.nih.gov/ij /
R language	The	R	https://www.r-
	Foundation		project.org/

Supplementary Table 5. Probes for mRNA expression related to Key resource table.

DESCRIPTION	IDENTIFIER
Mouse $\beta 2m$	Cat#:Mm00437762_m1
Mouse Arg1	Cat#:Mm00475988_m1
Mouse Il9r	Cat#:Mm00434313_m1
Human B2M	Cat#:Hs06637353_s1
Human IL6	Cat#:Hs00174131_m1
Human IL9R	Cat#:Hs01108522_m1